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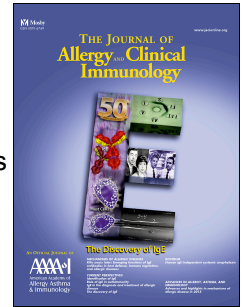
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Janus kinase 2 activation mechanisms revealed by analysis of suppressing mutations

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1 Abstract

2 **Background:** Janus kinases (JAK1–3, TYK2) mediate cytokine signals in the regulation of
3 hematopoiesis and immunity. JAK2 clinical mutations cause myeloproliferative neoplasms
4 and leukemia and the mutations strongly concentrate in the regulatory pseudokinase
5 domain, JAK homology 2, JH2. Current clinical JAK inhibitors target the tyrosine kinase
6 domain and lack mutation- and pathway-selectivity.

7 **Objective:** To characterize mechanisms and differences for pathogenic and cytokine-
8 induced JAK2 activation to enable design of novel selective JAK inhibitors.

9 **Methods:** Systematic analysis of JAK2 activation requirements using structure-guided
10 mutagenesis, cell signaling assays, microscopy, and biochemical analysis.

11 **Results:** Distinct structural requirements identified for activation of different pathogenic
12 mutations. Specifically, the predominant JAK2 mutation V617F is the most sensitive to
13 structural perturbations in multiple JH2 elements (C helix (α C), SH2-JH2 linker and ATP-
14 binding site). In contrast, activation of K539L is resistant to most perturbations. Normal
15 cytokine signaling shows distinct differences in activation requirements: JH2 ATP-binding
16 site mutations have only a minor effect on signaling, while JH2 α C mutations reduce
17 homomeric (JAK2-JAK2) EPO signaling, and almost completely abrogate heteromeric
18 (JAK2-JAK1) IFN γ signaling, potentially by disrupting a dimerization interface on JH2.

19 **Conclusions:** These results suggest that therapeutic approaches targeting the JH2 ATP-
20 binding site and α C could be effective in inhibiting most pathogenic mutations. JH2 ATP-
21 site targeting have potential for reduced side-effects by retaining EPO and IFN γ functions.
22 Simultaneously, however, we identify the JH2 α C interface as a potential target for
23 pathway-selective JAK inhibitors in diseases with unmutated JAK2, thus providing new
24 insights for the development of novel pharmacological interventions.

25 **Keywords**

26 Janus kinase, JAK2 V617F, cytokine signaling, myeloproliferative neoplasm, kinase
27 activation, drug design.

28 **Abbreviations**

29 JAK, Janus kinase

30 GOF, gain-of-function

31 MPN, myeloproliferative neoplasm

32 JH, JAK homology

33 Introduction

34 Janus kinases (JAKs) are non-receptor tyrosine kinases critically involved in cellular
35 signaling, regulating the immune system, development, differentiation, and growth ¹.
36 Signaling through JAKs are numerous proinflammatory cytokines, including interleukins
37 (IL-2, IL-3, IL-4, IL-6, IL-9, IL-12, IL-13, IL-15, IL-23, and granulocyte-macrophage colony
38 stimulating factor (GM-CSF), making JAK inhibition a tempting drug target for the
39 treatment of inflammatory diseases ². Similarly, aberrant signaling caused by activating
40 gain-of-function (GOF) mutations in JAKs underlie multiple neoplastic diseases, including
41 myeloproliferative neoplasms ³. Indeed, the recent advent of JAK inhibitors for the
42 treatment of both of these disease groups has made understanding the mechanisms of
43 JAK-STAT signaling highly relevant to the clinical immunologist ⁴.

44 JAKs associate with type I and type II cytokine receptors and mediate cytokine signals
45 from activated receptors to signal transducers and activators of transcription (STATs),
46 which upon phosphorylation by JAKs, move to the nucleus to activate transcription. The
47 four JAKs in mammals (JAKs 1–3, TYK2) signal at homodimeric (JAK2) or heterodimeric/-
48 oligomeric (all JAKs) receptors and consist of four domains (N-to-C): a 4.1-band, ezrin,
49 radixin, moiesin (FERM) domain, a Src homology 2 (SH2)-like domain, a pseudokinase
50 domain (JAK homology 2, JH2), and a protein tyrosine kinase domain (JH1). FERM-SH2
51 mediate association to cytokine receptors ⁵. JH2 serves a dual role; it inhibits the tyrosine
52 kinase activity of JH1 in the basal state, and is required for full activation upon cytokine
53 stimulation ⁶⁻⁹.

54 JH2 is a mutational hotspot for clinical JAK mutations. The somatic JAK2 V617F mutation
55 in exon 14, for example, causes ligand-independent JAK2 activation and underlies >95%
56 of polycythemia vera and >50% of essential thrombocythemia and primary myelofibrosis
57 cases ¹⁰. Other JAK2 GOF mutations are located in JH2 in exon 12 (residues 506–547,

including K539L), exon 16 (including R683S/G), and some in JH1³. JH2 GOF mutations in other JAKs cause leukemias and loss-of-function mutations cause immune deficiencies³, highlighting the dual regulatory role of JH2^{6,7,11}. Recent structural information of the JH2-JH1 interaction explains the inhibitory function of JH2^{9,12}. In this interaction, the C helix (α C) side of JH2 binds to JH1 in a front-to-back orientation (Figure 1 A) leading to conformational restriction of JH1 and inhibition of kinase activity¹².

While multiple GOF mutations lie in the JH2-JH1 interface, disruption of the JH2-JH1 interaction alone does not fully explain the high activation potential of all GOF mutations—including JAK2 V617F or JAK2 K539L. We speculate that these mutations utilize the known, but molecularly incompletely characterized, stimulatory function of JH2 to activate JAK2.

Current clinical JAK inhibitors used to treat diseases caused by JAK2 GOF mutations target JH1 and thus do not distinguish between mutated and wild-type (WT) JAK2 and are unable to eradicate the disease. Furthermore, they frequently lead to anemia caused by suppression of normal erythropoietin (EPO) signaling due to inhibition of JAK2 WT functions¹³. In contrast, in inflammatory diseases, in which usually no JAK mutations are present (with rare exceptions, see e.g., ref¹⁴) current inhibitors are effective in approximately half of the patients, but also affect unwanted cytokine functions and show side-effects such as reactivation of viral infections and anemia⁴. Thus, there is a clinical need for more effective and selective JAK inhibitors able to discriminate between pathogenic and cytokine-induced signaling and/or discriminate between different types of JAK-mediated signaling pathways.

However, a potential paradigm shift in JAK inhibition is emerging, as molecular characterization of JH2 is suggesting an alternative approach and implies JH2 to be a valid

82 target for novel modulators of JAK activity¹⁵. JH2 harbors the majority of human
83 pathogenic JAK mutations, and we recently identified the JAK JH2 ATP-binding site as a
84 potential drug target by demonstrating that activation by the pathogenic JAK2 JH2 GOF
85 mutations K539L, V617F, and R683S is reliant on the stabilizing effect of ATP binding on
86 JH2¹⁶. Furthermore an ATP-competitive compound targeting TYK2 JH2 has been
87 demonstrated to efficiently and specifically inhibit cytokine signaling¹⁷.

88 Here, we provide a systematic analysis of the molecular basis for different JH2-targeting
89 intervention strategies. We identify distinct differences in activation mechanisms between
90 clinical JAK2 GOF mutations in terms of reliance on specific activating JH2 molecular
91 interfaces and JAK2-mediated receptor dimerization. Analysis of cytokine-induced
92 signaling shows differences in JH2 interface requirements between homodimeric (EPO)
93 and heterodimeric (Interferon γ , IFN γ) JAK2 activation. These results provide novel
94 insights into pathogenic and cytokine induced JAK2 activation mechanisms that have
95 implications for development of mutant- and potentially pathway-preferring inhibitors.

Materials and Methods

See Supplementary material for full details of Materials and Methods. Briefly, for immunoblotting and luciferase reporter assays, JAK2-deficient γ 2A human fibrosarcoma cells¹⁸ were transfected with the designated combination of human JAK2-HA, human HA-EPOR (both in pCIneo), and human STAT5-HA (in pXM) using FuGENE HD (Promega) for 24–48 h. For reporter assays, a Firefly luciferase reporter plasmids for STAT5 (Spi-Luc¹) or STAT1 (IRF-GAS¹⁹) were added along with a constitutively expressing *Renilla* luciferase plasmid. Cytokine stimulation was done in starvation medium without FBS for 30 min (for immunoblotting) or 5 h (for reporter assays) unless otherwise specified, with recombinant human EPO (Roche), or IFN γ (Peprotech). For immunoblotting, cells were washed with PBS, lysed in Triton X-100 lysis buffer, and complete lysates run on lab-made SDS-PAGE gels. Immunoblots were blocked with bovine serum albumin and incubated with primary antibodies: HA Tag (Aviva Systems Biology), phospho-JAK2 (Millipore), phospho-STAT5 (Cell Signaling), phospho-STAT1 (Cell Signaling), STAT1 (BD Biosciences), or actin (Millipore), and a mixture of goat-anti-rabbit and goat-anti-mouse DyLight secondary antibodies (both Thermo Fisher Scientific). Blots were read using an Odyssey CLx (LI-COR), and immunoblot signals quantified using Image Studio software (LI-COR) by manually assigning bands (See Supplementary Material and Figure S1). Reporter assays were detected using the DualGlo reporter assay kit (Promega) according to manufacturer's instructions and normalized to readings from wells of unstimulated cells transfected with JAK2-HA WT.

For qPCR analysis, γ 2A cells were transfected for 28 h, starved for 16 h, stimulated for 2 h with 10 U/ml EPO or 10 ng/ml IFN γ , and RNA extracted using TRI Reagent (Molecular Research Center) according to manufacturer's instructions. IRF1 gene expression was measured from reverse-transcribed total RNA using specific primers (5'-

121 GCATGAGACCCTGGCTAGAG-3' and 5'-CTCCGGAACAAACAGGCATC-3') and
122 normalized to the expression of TATA-box binding protein (TBP).

123 For *in vitro* kinase assays, recombinant JAK2 JH2-JH1 (residues 513–1132-6xHis) WT,
124 I559F, and E592R proteins were expressed in High Five insect cells (Thermo Fisher
125 Scientific) using the Bac-to-Bac expression system (Invitrogen) according to
126 manufacturer's instructions. Cells were lysed by freeze-thawing, clarified by centrifugation,
127 and recombinant proteins purified using Ni-NTA agarose (Qiagen) followed by size-
128 exclusion chromatography in a HiLoad 16/600 Superdex 75 pg column (GE Healthcare).
129 Protein concentrations were measured by Bradford assay (Bio-Rad) and enzymatic activity
130 determined with Lance Ultra kinase assay (PerkinElmer) under conditions recommended
131 by the manufacturer. Kinase reactions were performed in triplicate and results shown are
132 representative from 2–3 individual experiments.

133 For microscopy, cells were seeded on 35 mm glass bottom dish (MatTek), transfected with
134 JAK2-YFP fusion constructs (in pEGFP) or EPOR-YFP/EPOR-CFP (in pBOF²⁰) overnight
135 and starved for 8 h. Cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde
136 for 15 minutes at room temperature, washed, and kept in PBS at 4 °C before imaging on a
137 Zeiss LSM 780 laser scanning confocal microscope using a Plan Apochromat 63x/1.4 oil
138 immersion objective. FRET was monitored by acceptor photobleaching²¹ and FRET
139 efficiency was calculated from manually segmented cell membrane areas.

140 Results

141 Suppressing mutations reveal differences in activation mechanisms of GOF 142 mutations

143 Studies on JAK2 activation mechanisms have identified several mutations capable of
144 suppressing activation by pathogenic JAK2 GOF mutations (see Table 1, Figure 1, Figure
145 2). These mutations, termed here 'suppressing mutations', localize in JH2 α C (F595A), in
146 the C-terminus of the SH2-JH2 linker (F537A) and in the JH2 ATP-binding site (see Table
147 1). Recently Leroy et al. identified an additional residue in the outer face of JH2 α C (JAK2
148 E596) as an important link in the activation mechanism of V617F, but not of K539L,
149 R683G, or of T875N²². Notably, these suppressing mutations are functionally distinct from
150 mutations that completely destabilize JH2 structure (e.g. JAK2 F739R refs^{16,23} or deletion
151 of JH2 α G⁸), which mimic JH2 deletion resulting in increased basal activation and
152 irresponsiveness to cytokines.

153 The activation mechanisms and requirements of regulatory interfaces for different GOF
154 mutations and cytokine-induced JAK2 activation have not been systemically analyzed. We
155 thus set out to compare ligand-independent (pathogenic) and normal ligand-dependent
156 JAK2 activation using suppressing mutations in JAK2-deficient γ 2A fibroblast cells. We
157 focused on the three previously identified regulatory regions in JH2: the ATP-binding site
158 (JAK2 mutations I559F, G552A+G554A, or K677E)¹⁶, the outer face of JH2 including α C
159 (F595A, E596R)^{12,22-25}, and the C-terminus of the SH2-JH2 linker (F537A)²⁶. Additionally,
160 we tested a novel JH2 α C outer face mutation, E592R, in order to analyze the involvement
161 of the N-terminus of the JH2 α C. We further hypothesized that JH2 functions as a
162 structural linker between FERM-SH2 and JH1 and, when structurally sound, is able to
163 position JH1 for trans-autophosphorylation. To test this, we aimed to break up the putative
164 interaction between FERM-SH2 and JH2 by introducing V511R to disrupt the short β sheet

between the SH2-JH2 linker and the FERM F1-F2 loop (Figure 1). We also included JH1 α C outer face mutations (E896A+E900A) analogous to the JH2 mutations E592R/E596R to test the function of JH1 α C as a potential interaction interface.

We analyzed activation by three different pathogenic GOF JAK2 mutations predicted to have differing activation mechanisms (Figure 2): V617F (exon 14), which has been suggested to alter the conformation of the SH2-JH2 linker and thus indirectly affect the inhibitory JH2-JH1 interaction^{12,26}; R683S (exon 16), which is predicted to activate primarily by breaking the inhibitory JH2-JH1 interaction^{9,12}; and K539L (exon 12), which lies at the N terminus of JH2, and thus might also affect the SH2-JH2 linker, but whose activation mechanism has not been studied in detail.

In accordance with previous reports^{16,22,24,25} we found that ligand-independent JAK2 JH1 activation loop (Y1007-Y1008, pJAK2) hyperphosphorylation caused by V617F is suppressed by JH2 ATP pocket and α C mutations (Figure 2 A and B, first panel). V617F-induced pJAK2 is also suppressed by V511R, suggesting that the activation mechanism of V617F requires correct linking of JH2 to SH2. However, JAK2V617F activation is not sensitive to perturbation of JH1 α C (Figure 2 A and B, first panel). Downstream pSTAT1 analysis correlated with pJAK2 levels. Effects of suppressing mutations on STAT5 activation were analyzed in reporter assays with EPOR-HA coexpression (Figure 2 D), where the inhibition profile correlated with pJAK2 and pSTAT1 analysis with strongest inhibition with α C mutations and F537A.

Activation by R683S was sensitive to all suppressing mutations in pJAK2 and pSTAT1 analysis as well as in STAT5 transcriptional activation, and the ATP-binding site mutants showed slightly more suppression than mutations in α C. Interestingly, K539L was clearly the most resistant to suppression, and only the α C mutation F595A strongly suppressed

189 K539L in pJAK2, pSTAT1, and STAT5 activation. JH2 ATP-binding site mutations affected
190 mainly JAK2 phosphorylation. These data suggest a distinct activation mechanism for
191 K539L over V617F and R683S.

192 Taken together, these results indicate that interactions involving JH2 are critical for
193 hyperactivation of all JAK2 GOF mutants, but that the specific JH2-mediated interactions
194 differ between the GOF mutations.

195 **The effect of suppressing mutations on cytokine activation**

196 Cytokine stimulation titrations with JAK2-HA V617F+suppressor double-mutant constructs
197 showed that even strong suppression of basal V617F-induced activity did not inhibit
198 cytokine-induced STAT5 transcriptional activation for EPO or STAT1 activation for IFN γ
199 (Figure 3 A and B, respectively). Rather, the most potent suppressor mutations (F537A
200 and all α C mutations) restored EPO sensitivity to be indistinguishable from JAK2 WT
201 (Figure 3 A). For IFN γ , cytokine sensitivity was also restored, which was further
202 corroborated with qPCR of induction of expression of the IFN γ -responsive gene *Interferon*
203 *regulatory factor 1* (IRF1). Interestingly, however, IFN γ -induced STAT1 activation with α C
204 and F537A mutations with V617F were lower than with JAK2 WT (Figure 3 B and C)
205 suggesting potential specific involvement of these regions in IFN γ signaling.

206 Previous work has suggested that suppressing mutations do not inhibit cytokine-induced
207 signaling in a JAK2 WT background^{16,22,24}, but detailed analysis of sensitivity to different
208 modes of JAK2-mediated signaling (homo- vs. heterodimeric) has been lacking. We thus
209 analyzed cytokine-induced JAK2 activation using the mutation panel in a JAK2 WT
210 background. Immunoblot analysis of JAK2-mediated STAT5 phosphorylation on
211 homodimeric EPO receptor showed that, despite lower basal signaling activity, EPO-
212 induced signaling was preserved in suppressing mutations (Figure 4 A), and JH2 ATP-

213 binding site mutations were virtually identical to JAK2 WT in their response to EPO. F595A
214 and E592R in α C and F537A, however, showed diminished EPO-induced STAT5
215 phosphorylation (Figure 4 A). Reporter assays showed similar results, albeit with
216 differences even more pronounced (Figure 4 C).

217 Strikingly, the same JH2 α C and SH2-JH2 linker mutations practically abolished
218 heteromeric JAK2-JAK1-mediated STAT1 phosphorylation upon IFN γ stimulation (Figure 4
219 B), while JH2 ATP-site mutations were again indistinguishable from JAK2 WT. In
220 accordance with pSTAT1-immunoblot data, IFN γ -induced STAT1 transcription activity was
221 almost completely abrogated for all α C mutations (including E596R) and F537A, whereas
222 JH2 ATP-site mutations and V511R showed no significant decrease (Figure 4 D). *IRF1*-
223 qPCR further corroborated these results with E592R and I559F (Figure 4 E).

224 We also measured STAT1 activation with longer IFN γ stimulation times to estimate rule
225 out simply delayed signaling kinetics²⁷, and found no activation of STAT1 with E592R or
226 F537A mutants even at long time-scales (Figure S2 D).

227 **Characterization of JAK2 GOF activation mechanisms by suppressor mutations**

228 JAK2 V617F hyperactivation relies on the interaction with cytokine receptors but the
229 underlying mechanisms are still elusive²⁸⁻³⁰. Disruption of FERM and receptor binding of
230 JAKs to receptors is a potential mechanism of suppression²⁸, and it has been suggested
231 that some JAK3 JH2 mutations (including a JH2 ATP-binding site mutation) could affect
232 subcellular localization of JAK3³¹. We assessed subcellular localization of our suppressing
233 mutations by imaging JAK2-YFP fusion proteins, but found no effect for either mutation on
234 subcellular localization, either with or without added EPOR-HA (Figure 5 A). In contrast,
235 JAK2-YFP with Y119E, known to cause dissociation from receptors^{29,32}, showed
236 exclusively cytoplasmic JAK2 (Figure 5 A).

237 We speculated that mutation-induced JAK2-receptor dimerization is part of the activation
238 mechanism of JAK2 GOF mutations. We thus analyzed whether suppressing mutations
239 directly affect the propensity of JAK2 to dimerize on receptors. Using a FRET-based
240 EPOR-CFP/YFP receptor dimerization assay in the JAK2-deficient γ 2A fibroblast cell line
241 (lacking endogenous EPOR expression) along with our JAK2-HA mutant constructs
242 showed that E592R significantly reduces basal JAK2-EPOR dimerization (Figure 5 B).
243 I559F, in contrast, showed a slight increase in dimerization, but this was within
244 experimental noise and not significant.

245 To directly assess whether decreased dimerization propensity also translates to decreased
246 receptor-mediated JAK2 activation, we assessed basal activation of otherwise wild-type
247 JAK2. EPOR-HA overexpression (known to induce ligand-independent activation ¹⁶)
248 alongside expression of JAK2 mutants showed that all suppressing mutations, irrespective
249 of their mode of action, suppress EPOR-induced JAK2 activation (Figure 5 C), suggesting
250 that other mechanisms beyond lowering of dimerization propensity (as shown for E592R
251 (Figure 5 B), and potentially also true for other α C mutations) are likely at play to explain
252 the mode of action of suppressing JH2 ATP-binding site mutations. We thus measured
253 whether mutating the JH2 ATP-binding site or α C directly affects the enzymatic activity of
254 JH1. Indeed, kinase assays with recombinant JAK2 JH2-JH1 fragments *in vitro* showed
255 unchanged $K_{m, ATP}$ values for both JH2 mutations I559F and E592R, but lowered kinase
256 reaction catalysis rates (k_{cat}) for I559F (Figure 5 D).

257 Taken together, these data suggest that the mechanisms of suppression of JH2 ATP-
258 binding site and α C mutations are different. Inhibiting ATP binding to JH2 directly lowers
259 catalytic activity of JH1, potentially through lowering the stability of the JH2 α C ¹⁶, and thus
260 strengthening the JH2-JH1 interaction ¹². Altering the outer face charge of α C directly

261 (e.g., with E592R), on the other hand, inhibits the propensity of JAK2 to dimerize and
262 thereby hinders JH1 activity by suppressing trans-autophosphorylation (Figure 5 B, D).

Discussion

The molecular mechanisms of JAK activation by cytokine or mutation have long been elusive and here we have performed a systematic analysis of JAK2 activation mechanisms using structure-guided mutagenesis. Our results shed light on not only the mechanism of cytokine-independent JAK2 activation, but also identify a previously unknown interface on JH2 involved in JAK2-mediated receptor dimerization and needed especially for heteromeric JAK signaling.

Our results enable grouping of activating JAK2 mutations based on their requirements for distinct structural elements and thus activation mechanisms. Both V617F and R683S were sensitive to mutations affecting the JH2 ATP binding site and α C, albeit their suppressing effects showed differences, i.e., α C mutations completely abrogated V617F but not R683S, while JH2 ATP-binding site mutations showed similar suppression of both. The effect of suppressing mutations were more pronounced at pJAK2 and pSTAT1 than on STAT5 reporter assays which may reflect technical differences (e.g., stability of luciferase), or be indicative of signal amplification in the JAK-STAT pathway. Previously, we have shown that a suppressing JH2 ATP-binding site mutation reverts the increased hematocrit in a mouse V617F MPN model¹⁶.

The resistance of K539L compared to V617F and R683S to suppressing mutations is interesting, since K539 and V617 reside near each other in the JH2 structure (Figure 1), and suggests a distinct activation mechanism for K539L. R683S likely activates through breaking the autoinhibitory interface resulting in increased conformational freedom and activation of JH1. This freed JH1 does, however, still rely on correct JH2-mediated positioning for ligand-independent activation, as well as JAK2-mediated receptor dimerization (see V511R and α C mutations, respectively in Figure 2). In contrast, K539L is unlikely to simply interfere with the autoinhibitory interaction, and our inhibitory profile

analysis is consistent with a more direct activation mechanism of K539L, potentially involving direct activation of JH1, e.g., through interaction with K857 on JH1²². Consistent with previous reports, K539L is effectively inhibited only by F595A, which is known primarily for participating in stabilizing interactions in JH2 α C in the context of V617F hyperactivation^{24,25}, but which has been suggested to alter the stability of the JH2 α C also more broadly.

For V617F, our results show complete inhibition by α C mutations including E592R, which our FRET-data indicate to interfere with JAK2-mediated receptor dimerization (Figure 5 B). We thus hypothesize that V617F activates JAK2 mostly by enhancing the propensity of JAK2 to dimerize on a receptor. This is in line with previous reports with recombinant JAK2 and TYK2 JH2-JH1 fragments, which showed only modest activation of kinase activity with the JAK2 V617F or analogous TYK2 V678F mutation in an isolated *in vitro* setting, which does not include JAK-mediated receptor dimerization effects^{9,33}. Our mutagenesis data furthermore suggests, that the dimerization interface directly includes the JH2 α C, with E592 (and probably E596) involved.

Our analysis of suppressing mutations in an otherwise wild-type background shows that, contrary to previous reports^{16,22,24}, suppressing mutations do affect JAK2 WT activity by lowering both basal (Figure 5 C), as well as ligand-dependent activation (Figure 4). Interestingly, quantitative comparison of potency of individual suppressing mutations to inhibit activation by V617F and cytokine reveals that these two correlate clearly (Figure S3). The correlating suppression of ligand-dependent and -independent JAK2 activation suggests that the same JH2 interface (α C and C-terminus of SH2-JH2 linker) is used in both settings. Furthermore, imaging data of JAK2-YFP shows unaltered subcellular distribution of JAK2 carrying suppressing mutations (Figure 5 A) ruling out direct destabilization of JAK2/FERM as an explanation for suppression.

Our results also reveal that JH2 α C suppressing mutations most likely inhibit JAK2 activation by suppressing JAK2 dimerization (Figure 5 B), while JH2 ATP-binding site mutations exert their suppressing effects by directly affecting tyrosine kinase activity of JAK2 JH1 (Figure 5 D), potentially through partial destabilization of JH2 α C^{12,16}. We thus conclude, that V617F and R683S most likely activate JAK2 by increasing its propensity for dimerization, and that this is counteracted by suppressing mutations in the JH2 α C (E592R, E596R, F595A) and SH2-JH2 linker (F537A). For the case of R683S, which lies directly in the JH2-JH1 autoinhibitory interface (Figure 1 and refs^{9,12}), we speculate that weakening of the autoinhibitory interaction releases JH2, which relieves autoinhibition on JH1, as well as exposing the dimerization interface on JH2.

The molecular details of heteromeric JAK activation have remained largely unknown. Our analysis of cytokine-stimulation of JAK2 carrying suppressing mutations strikingly suggest that the same interface needed for activation by V617F or R683S by dimerization of (receptor-bound) JAK2, is crucial especially for heteromeric activation of JAK2. This finding refines earlier work that showed a critical role for JH2 in JAK activation^{7,11}. Previous studies have shown in several cytokine receptor systems that catalytic activity of both JAKs is not required for heteromeric JAK activation^{20,34}. For instance, in IFN γ signaling STAT1 does not require enzymatically functional JAK1³⁵, but does require the presence of JAK1 JH2³⁶. Pathway-specific JAK substructures have also been implicated in JAK2 JH1 for EPO signaling³⁷. Our results refine these findings by identifying the C-terminus of the SH2-JH2 linker and JH2 α C as critical for heteromeric JAK activation. However, whether the JH2 interface identified here participates in JAK2-JAK2 or JAK2-JAK1 dimers/multimers on IFNGR remains a topic for future research.

Currently available JAK inhibitors show beneficial clinical responses, but there is a clear need for more effective, optimally disease-selective drugs with less side-effects. The key

338 question for this goal is to understand the differential mechanisms defining pathogenic and
339 different cytokine-induced activation modes. Our results presented here provide insights
340 into these questions and identify specific regions in JH2 that are differentially required for
341 JAK2 activation in different contexts. These findings pave the way for the design of novel,
342 potentially mutant and/or pathway-selective pharmacological compounds.

343

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487

488 Figure Legends

489 **Figure 1: JAK2 domain structure.** A: Structures of JAK2 FERM-SH2 (left, PDB: 4Z32)
490 with model of EPOR JAK2-binding peptide shown in dark blue (modelled based on
491 Interferon λ 1 receptor (IFNLR1) peptide bound to JAK1 FERM-SH2, PDB: 5L04), and
492 JAK2 JH2-JH1 inhibitory interaction¹². Right: JAK2 JH2-JH1 top view. B: Domain structure
493 of JAK2. See also Table 1.

494 **Figure 2: Suppressing JAK2 mutations reveal distinct activation mechanisms for**
495 **different JAK2 gain-of-function (GOF) mutations.** A: Representative immunoblots of
496 whole-cell lysates from JAK2-deficient γ 2A cells transiently transfected with full-length
497 JAK2-HA mutants as indicated. pJAK2, JAK2 activation loop phosphorylation
498 JAK2(Y1007/1008); pSTAT1, STAT1(Y701) phosphorylation. GGAA, G552A+G554A.
499 EEAA, E896A+E900A. Experiment was repeated twice with similar results. B and C:
500 Quantification of immunoblots shown in A. a.u., arbitrary units. D: STAT5 reporter assay in
501 the presence of transfected EPOR-HA. Averages and standard deviations from triplicate
502 wells are shown as fold induction relative to unstimulated JAK2 WT. RLU, relative
503 luminescence units. All reporter experiments were repeated twice with similar results.

504
505 **Figure 3: Suppression of V617F activation by secondary mutations restores**
506 **cytokine sensitivity.** A: STAT5 reporter assay in the presence of transfected EPOR-HA.
507 B: IFN γ /STAT1 reporter. A and B as described for Figure 2. C: quantitative PCR (qPCR) of
508 IFN γ -induced *interferon regulatory factor 1* (IRF1). Averages and standard deviations from
509 two biological replicates each done in technical triplicates in qPCR are shown. Mutations
510 are color-coded by type as in Figure 2.

511 **Figure 4: Analysis of suppressing mutations in JAK2 WT background.** A and B:
512 Quantifications from immunoblots, see also Figure S2 A and B. C: STAT5 reporter assay

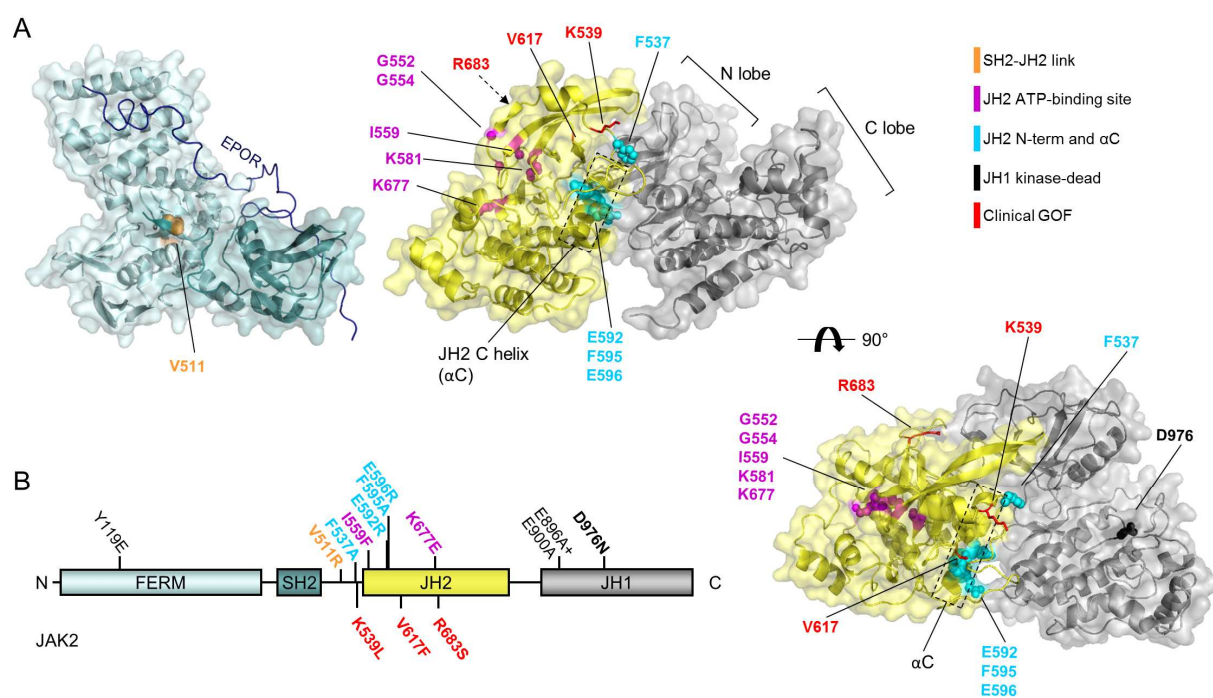
513 in the presence of transfected EPOR-HA. D: IFN γ /STAT1 reporter assay. C and D as
514 described for Figure 2. E: qPCR of *IRF1* expression as described for Figure 3. Wild-type
515 sample (WT) same as in Figure 3 C. Mutations are color-coded by type as in Figure 2.
516 Experiments were repeated twice with similar results.

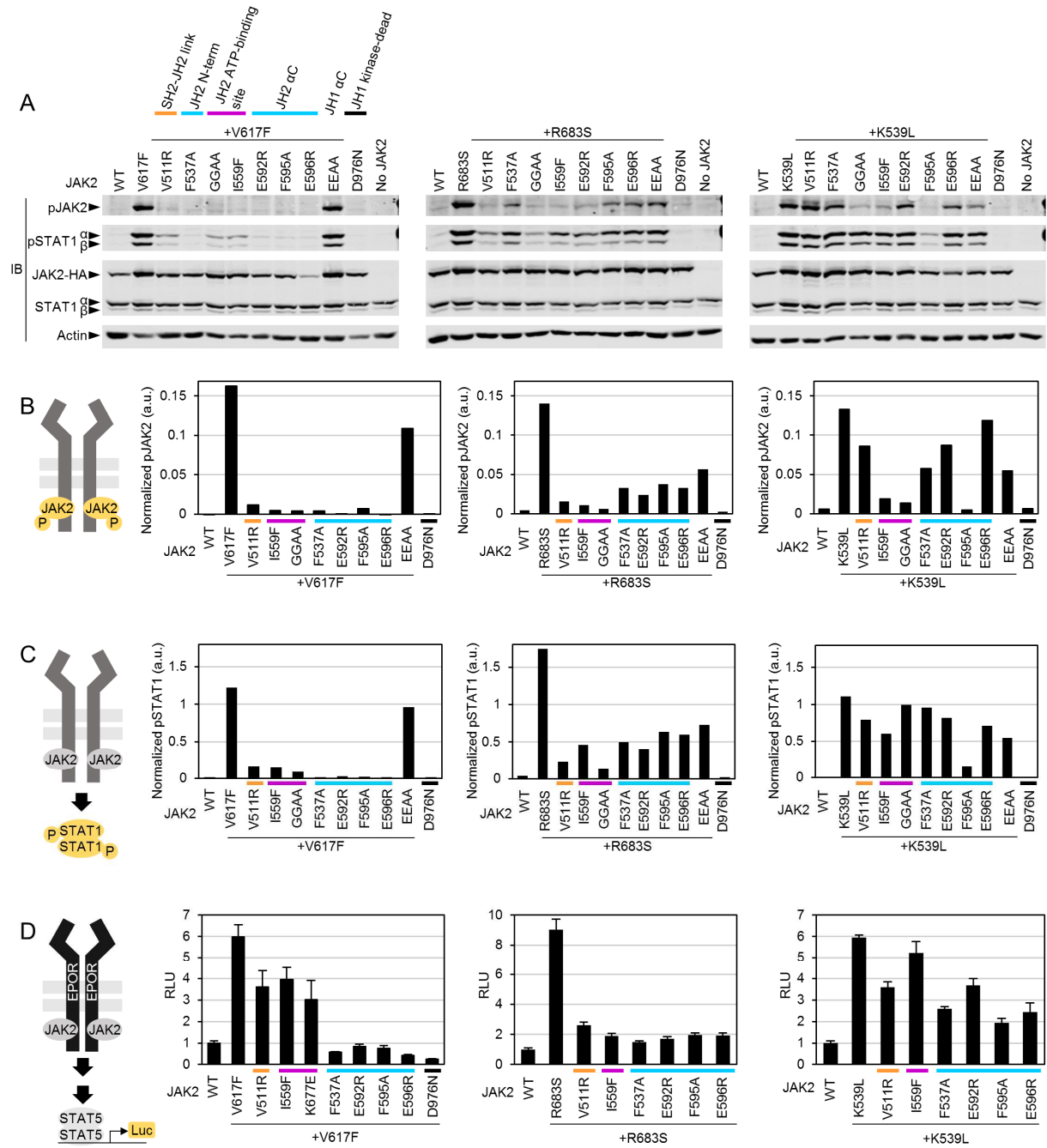
517 **Figure 5: Suppressing mutations localize correctly to the membrane, but**
518 **differentially affect dimerization of JAK2-EPOR and kinase activity of recombinant**
519 **JAK2 JH2-JH1.** A: Representative confocal microscopy micrographs of fixed γ 2A cells
520 expressing the indicated JAK2-YFP mutations. B: Analysis of basal JAK2-EPOR
521 dimerization. Normalized apparent FRET efficiency calculated from manually segmented
522 cell membranes as detailed in Materials and Methods. Number of individual cells analyzed
523 for each condition is indicated. Significance assessed by Student's t test (unpaired). n.s. =
524 not significant; * $p < 0.05$. C: Immunoblot analysis of whole-cell lysate from γ 2A cells
525 transiently transfected with the JAK2-HA constructs and EPOR-HA as shown. D: Kinase
526 assay with purified recombinant JAK2 JH2-JH1. Shown are averages and standard
527 deviation from triplicate measurements.

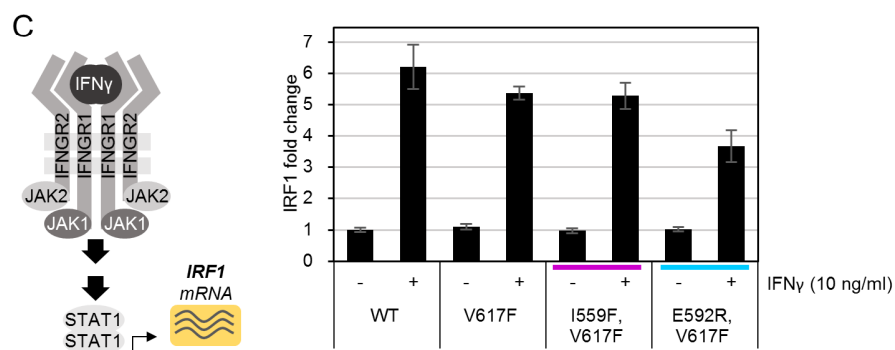
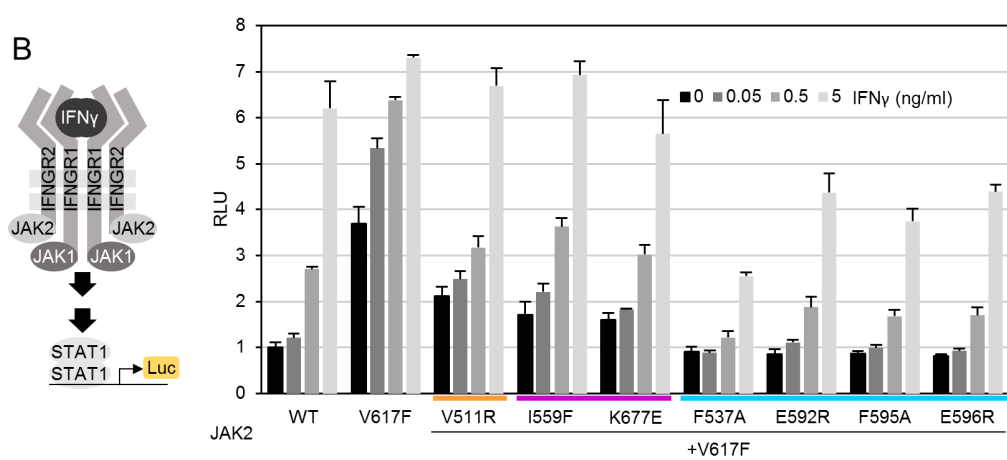
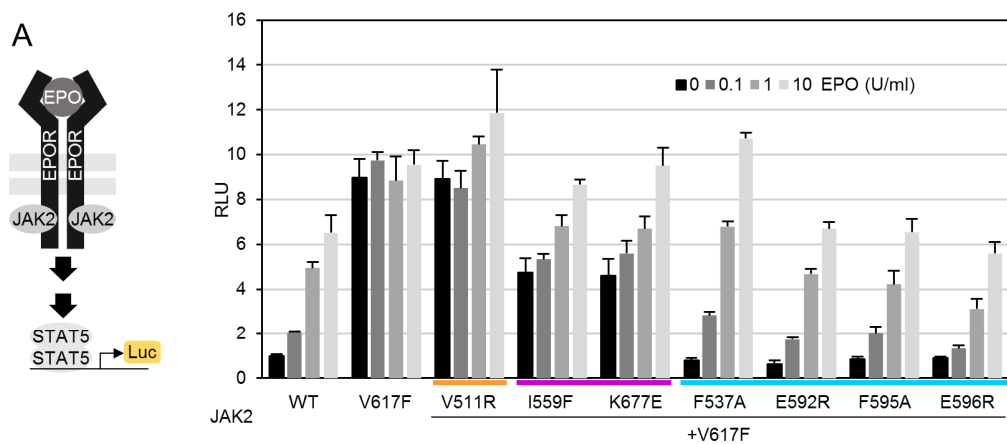
528 **Tables and Table Legends**

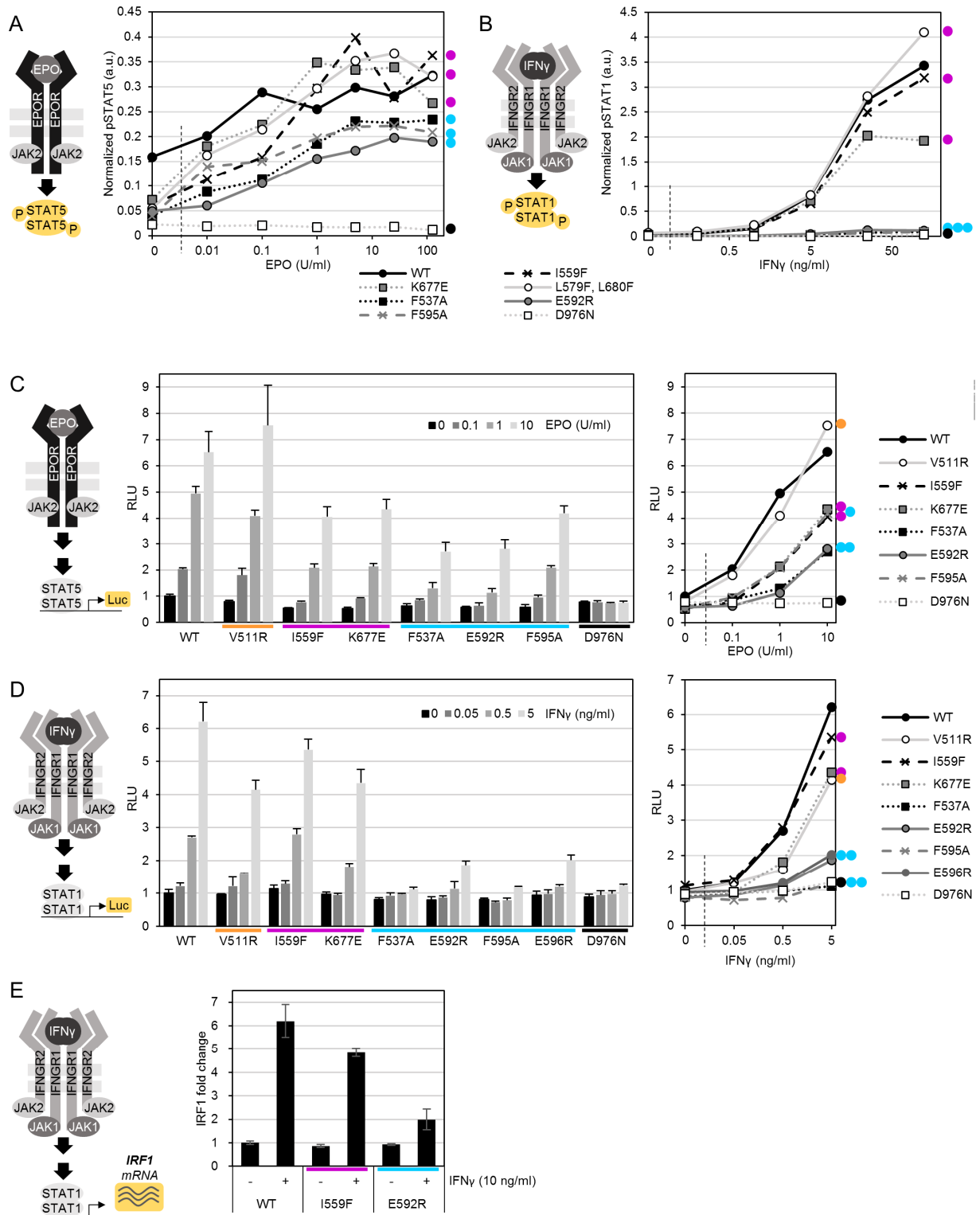
529 **Table 1:** Used JAK2 mutations and their presumed mode of action or experimental
 530 rationale. See also Figure 1.

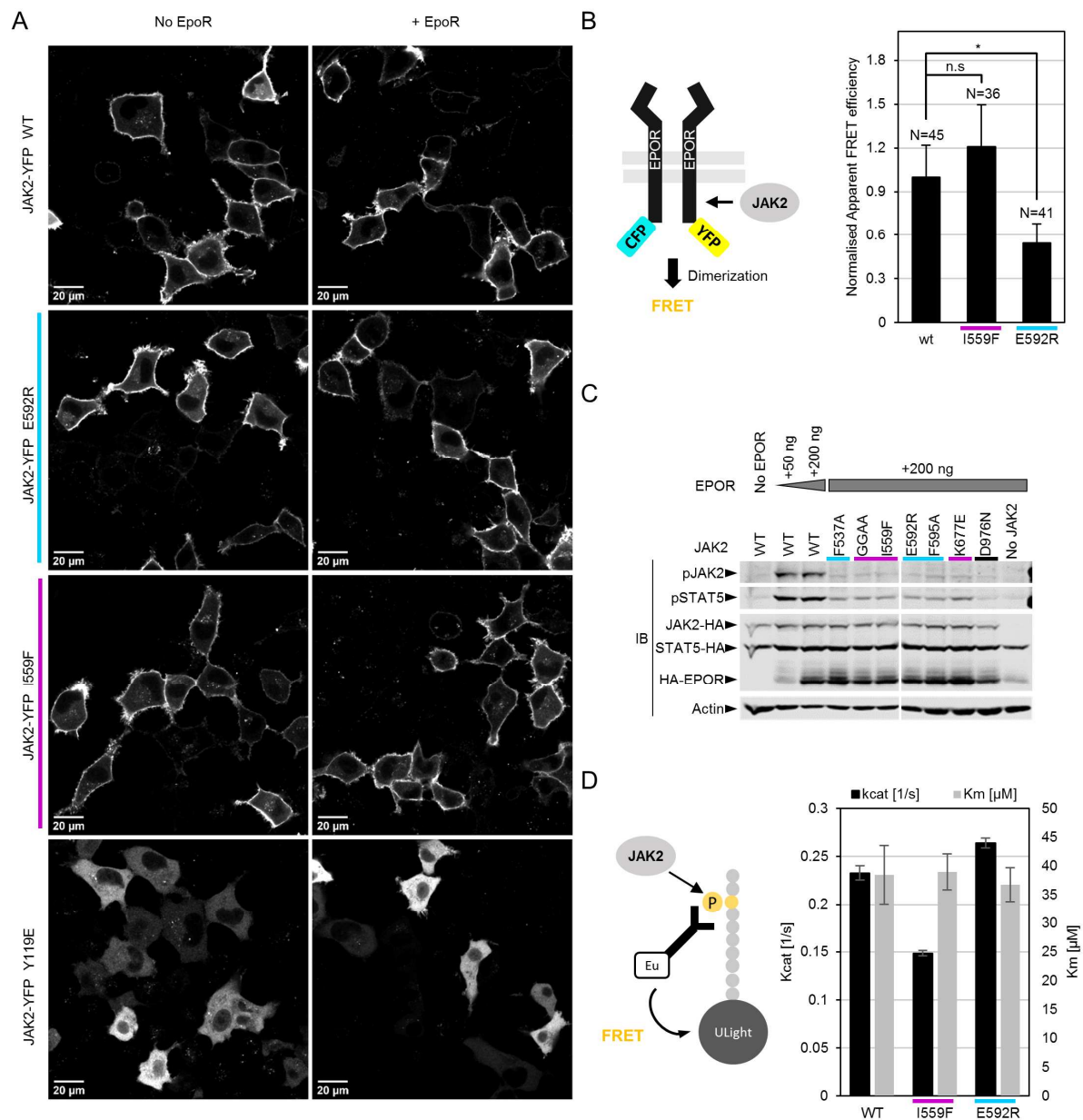
Mutation	Substructure	Rationale / mode of action	Reference
Y119E	FERM F1	Mimics Y119 phosphorylation. Previously reported to induce dissociation of JAK2 from receptor.	29,32
V511R	SH2	Designed to disrupt SH2-JH2 linker from FERM-SH2.	-
F537A	SH2-JH2 link	F537 proposed to stack with F595 in JAK2 JH2 WT. Known to inhibit V617F.	26
K539L	SH2-JH2 link	Activating by unknown mechanism. Causes PV.	38
G552A + G554A	JH2 β 1: Gly-rich loop	Designed to remove flexible glycines usually needed for ATP binding.	16
I559F	JH2 β 2	Designed to sterically inhibit ATP binding. Verified to inhibit ATP binding ¹⁶ .	16
K581A	JH2 β 3	Removes conserved β 3 lysine.	16,39
E592R	JH2 α C	Outer face of JH2 α C	12
F595A	JH2 α C	Inner face of JH2 α C. Known to inhibit V617F and others by potentially destabilizing JH2 and making space for F617 (ref ¹²).	12,23-25
E596R	JH2 α C	Outer face of JH2 α C. Known to inhibit V617F and others. Mechanism unknown.	22
V617F	JH2 β 4- β 5 loop	Activating, potentially by disturbing SH2-JH2 linker. Causes MPNs.	40-43
K677E	JH2 β 6- β 7 loop	Designed to inhibit ATP binding electrostatically. Verified to inhibit ATP binding.	16
R683S	JH2 β 7- β 8 loop	Activating, probably by breaking R683-D873 interaction over inhibitory JH2-JH1 interface. Causes ALL.	44,45
T875N	JH1 β 2- β 3 loop	Activating, mechanism probably similar to R683S. Causes AMKL.	46
L884P	JH1 β 3- α C loop	Activating by unknown mechanism. Homologous to JAK3 L857P found in ALL.	47
E896A + E900A	JH1 α C	Outer face of JH1 α C.	-
D976N	JH1 β 6- β 7 loop	D in HRD. Mutation is catalytically inactive (i.e., kinase dead).	-

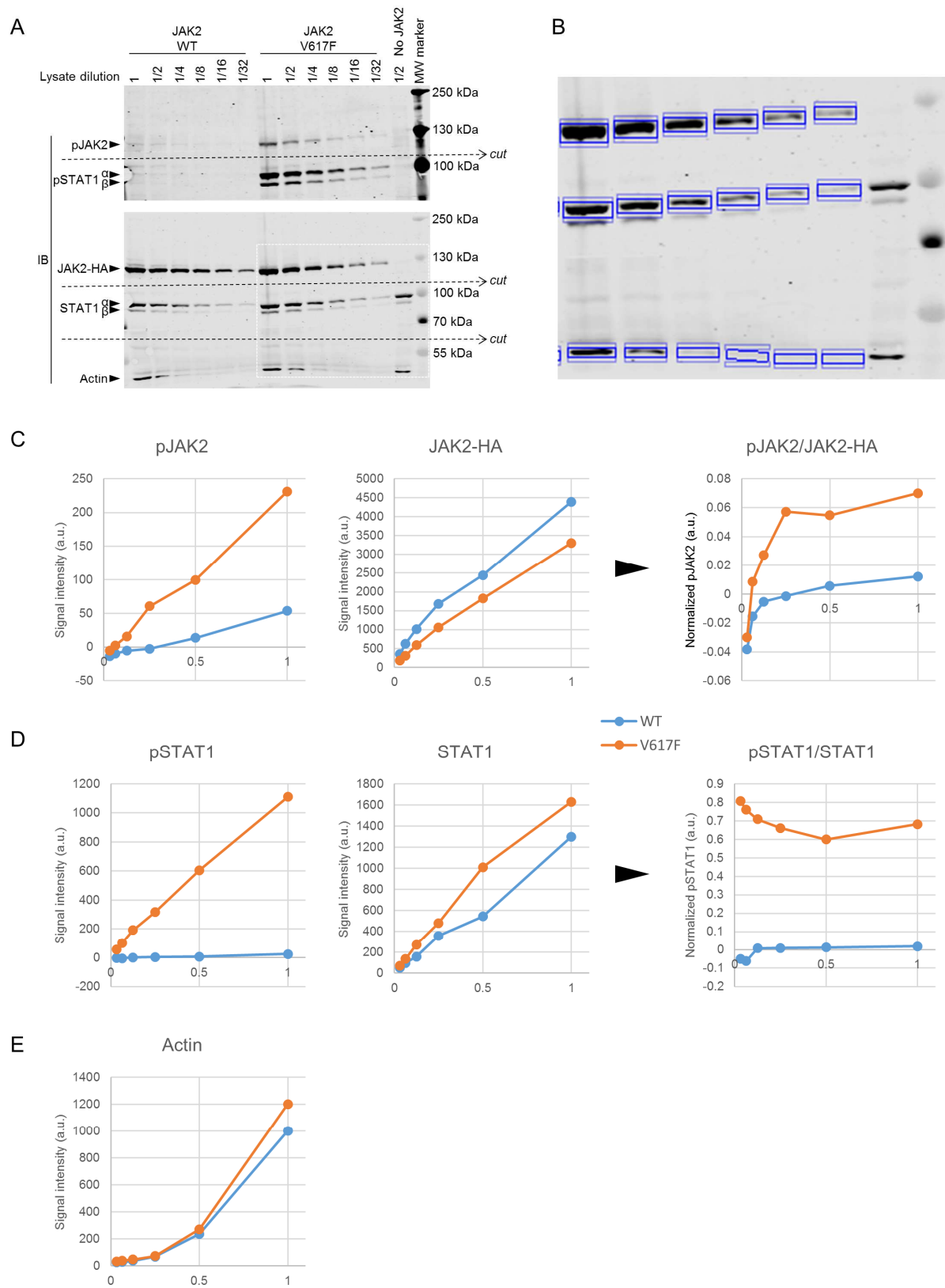


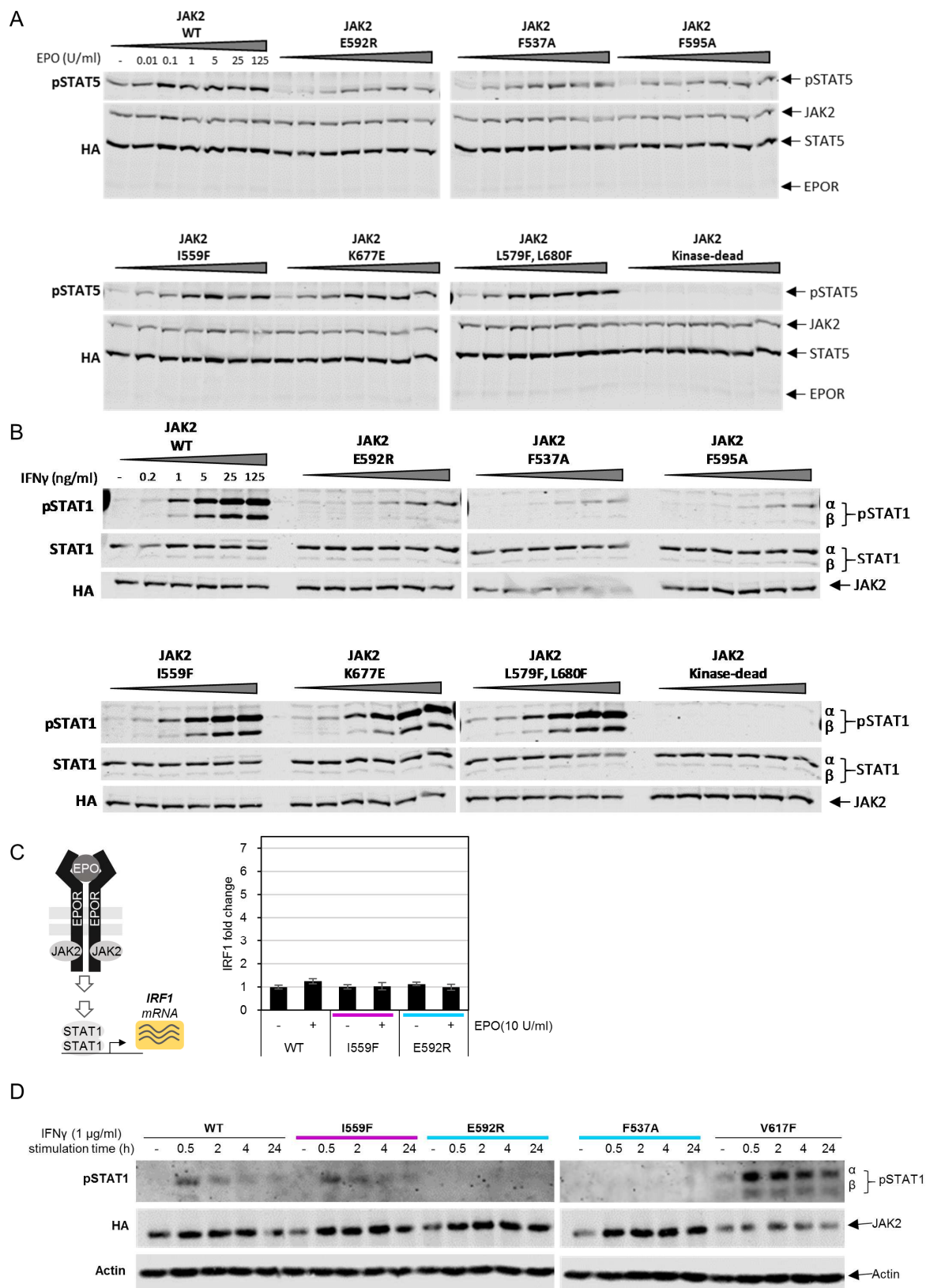


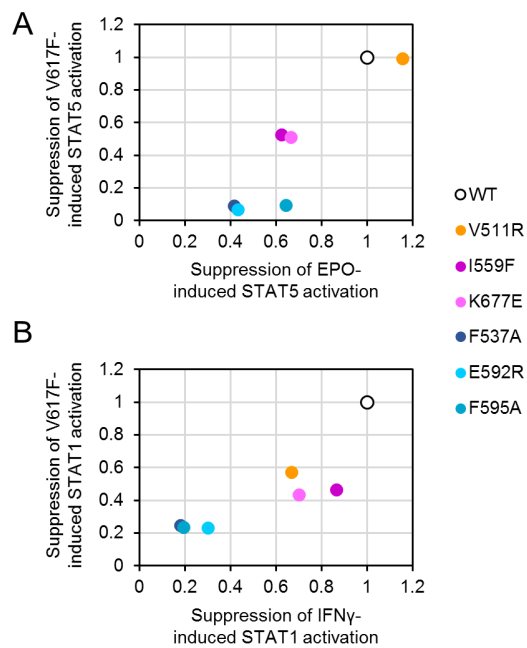












Supplementary

Materials and Methods

Plasmid constructs and mutagenesis. Full-length human JAK2 and erythropoietin receptor (EPOR) were cloned into pCIneo expression vector using Sall-NotI restriction sites. Full-length human STAT5A was in pXM vector. JAK2 and STAT5A were C-terminally hemagglutinin (HA)-tagged. EPOR was HA-tagged N-terminally after the signal peptide (between residues 30 and 31). Site-directed mutagenesis was performed using QuikChange (Agilent) according to manufacturer's instructions, and verified by Sanger sequencing. For luciferase reporter assays, Firefly luciferase reporter constructs for STAT5 (Spi-Luc¹) or STAT1 (IRF-GAS²) was used together with a constitutively expressing *Renilla* luciferase plasmid. For analysis of subcellular localization of JAK2 mutants, JAK2-YFP fusion constructs were made by cloning JAK2 without stop codon to pEGFP vector using Sall-XmaI restriction sites. The YFP variant (mCitrine – gift from Robert Campbell, Michael Davidson, Oliver Griesbeck, Roger Tsien; Addgene plasmid #54594) was cloned to XmaI-NotI restriction sites resulting in the JAK2-YFP fusion construct. A short flexible linker (amino acids RSIAT) was also inserted between JAK2 and YFP during cloning. EPOR FRET reporter constructs were created by fusing CFP or YFP to the N-terminus of EPOR truncated after residue 340 by overlap extension PCR. The fused EPOR-YFP and EPOR-CFP fragments were cloned to the bidirectional pBOF-vector³ to ensure equal expression of the EPOR FRET pair. Cotransfection with pTetOn vector and doxycycline (0.1 µg/ml) was used during transfection to induce expression of FRET reporter constructs.

Mammalian cell culture. JAK2-deficient γ2A human fibrosarcoma cells⁴ were cultured using standard culturing conditions in DMEM (Lonza) supplemented with 10% fetal bovine serum (FBS, Sigma), 2 mM L-glutamine (Lonza), and antibiotics (0.5% Pen/Strep, Lonza).

For transfection, cells were seeded on 6-, 12-, or 24-well tissue culture plates and transfected using FuGENE HD (Promega) according to manufacturer's instructions. Cells were transfected for 24–48 h and, where needed, cytokine stimulated in starvation medium without FBS for 30 min (for immunoblotting) or 5 h (for reporter assays) unless otherwise specified, with human recombinant EPO (NeoRecormon, Roche), or human recombinant IFN γ (Peprotech).

Immunoblotting. After transfection/stimulation, cells were washed with ice-cold PBS, and lysates collected in cold lysis buffer (50 mM Tris-Cl pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 2 mM vanadate, 8.3 μ g/ml aprotinin, 4.2 μ g/ml pepstatin, and 1 mM phenylmethanesulfonyl fluoride). Lysates were centrifuged and used directly for SDS-PAGE/immunoblotting or stored at -20 °C. Immunoblots were blocked with bovine serum albumin (BSA) and double-stained with the following diluted primary antibodies: HA Tag (1:2000, Aviva Systems Biology OAEA00009), phospho-JAK2 (1:1000, Tyr1007/1008, Millipore 07-606), phospho-STAT5 (1:1000, Tyr694, Cell Signaling 9351), phospho-STAT1 (1:1000, Tyr701 (D4A7), Cell Signaling 7649), STAT1 (1:1000, BD Biosciences 610116), or actin (1:1000, Millipore MAB1501R). Signals were detected using a mixture of goat-anti-rabbit (DyLight 680) and goat-anti-mouse (DyLight 800, Thermo Scientific) secondary antibodies (both at 1:5000 dilution) and read using an Odyssey CLx (LI-COR). Quantification of immunoblot signals was done using Image Studio software (LI-COR) by manually assigning bands to be quantified. For STAT1, only the larger isoform (STAT1 α) was assessed. Control experiments were carried out to ensure that signals were within the quasi-linear range of the detection method (See Figure S1).

Luciferase reporter assays. For reporter assays, cells were seeded on 6 or 12-well plates, transfected overnight, transferred onto 96-well plates, let attach overnight, and starved/stimulated for 5 hours, after which signals were detected using the DualGlo

reporter assay kit (Promega) according to manufacturer's instructions. Luminescence was read on an EnVision multiplate reader (Perkin Elmer), and results calculated as Firefly luciferase luminescence divided by *Renilla* luciferase luminescence and normalized to readings from wells of unstimulated cells transfected with JAK2 WT.

RNA isolation and qPCR. For quantitative PCR (qPCR) analysis, γ 2A cells were transfected as described above for 28 h, starved for 16 h, stimulated for 2 h with 10 U/ml EPO or 10 ng/ml IFN γ , and then RNA extracted using TRI Reagent (Molecular Research Center) according to manufacturer's instructions. Total RNA (0.2 μ g) was reverse transcribed using M-MuLV reverse transcriptase (Thermo Scientific) according to manufacturer's instructions. qPCR reactions with the cDNAs were made using HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne) and primers specific for IRF1 which is an IFN γ responsive gene (Primers- 5'-GCATGAGACCCTGGCTAGAG-3' and 5'-CTCCGGAACAAACAGGCATC-3'). The qPCR reaction was performed using Bio-Rad CFX-384 Real-Time PCR detection system and the gene expression was quantified using comparative C(T) method by normalizing to the expression of TATA-box binding protein (TBP).

Recombinant protein production, purification, and *in vitro* kinase assay.

Recombinant human JAK2 JH2-JH1 (513–1132-6xHis) WT, I559F, and E592R proteins were expressed in High Five insect cells (Thermo Fisher Scientific) using the Bac-to-Bac baculovirus expression system (Invitrogen) according to manufacturer's instructions. After protein expression (10% P3 virus, 48 h, 27 °C), the cells were collected by centrifugation, resuspended in lysis buffer containing 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 20 mM imidazole supplemented with phosphatase and protease inhibitors (100 mM sodium orthovanadate, 100 mM PMSF, 10 μ g/ml pepstatin A), and lysed by applying two freeze-thaw cycles. The lysates were clarified by centrifugation and recombinant

proteins were purified using Ni-NTA agarose (Qiagen) and size-exclusion chromatography in HiLoad 16/600 Superdex 75 pg column (GE Healthcare) equilibrated in 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 0.5 mM TCEP. Protein concentrations were determined by Bradford assay (Bio-Rad) according to manufacturer's instructions. Enzymatic activity of JAK2 JH2-JH1 WT and mutant proteins was determined by time-resolved (TR-)FRET-based Lance Ultra kinase assay (PerkinElmer) under conditions recommended by the manufacturer. Kinase reactions: 100 nM tyrosine kinase substrate ULight™-poly GT, recombinant JAK2 JH2-JH1 WT (60 pM), I559F (150 pM) or E592R (60 pM), 2 nM Eu-labeled anti-phospho antibody, and ATP concentration range of 0–1000 μ M, were set up in kinase buffer (50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1 mM EGTA, 0.05% BSA, 0.01% Brij-35, and 0.5 mM TCEP) on 384-plates (AlphaPlate-384 SW, PerkinElmer). Substrate phosphorylation was detected by measuring TR-FRET (ex. 320 nm, em. 665 nm) in 5 min intervals for 2 h at room temperature using EnVision Multilabel plate reader (PerkinElmer). Activity parameters k_{cat} and K_m were calculated by fitting reaction velocity vs. ATP concentration in GraphPad Prism (GraphPad Software). Kinase reactions were performed in triplicate and results shown are representative data from 2–3 individual experiments.

Microscopy and FRET assay to quantify JAK2 dimerization. For microscopy, cells were seeded on 35 mm glass bottom dish (MatTek), transfected overnight as described above and starved for 8 h. Cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde for 15 minutes in room temperature, washed and kept in PBS at 4 °C before imaging. The cells were imaged using a Zeiss LSM 780 laser scanning confocal microscope using a Plan Apochromat 63x/1.4 oil immersion objective and images were acquired with 458 nm and 514 nm excitation laser for CFP and YFP, respectively. Fluorescence was monitored between 465–500 nm for CFP and 525–640 nm for YFP with

32-channel QUASAR GaAsP PMT array detector. FRET was monitored by acceptor photobleaching⁵ using 514 nm laser and the images were processed with ImageJ. Cell images were sorted based on the expression levels by measuring acceptor fluorescence before photobleaching (independent of FRET) and only cells with approximately equal levels of expression for different constructs were used for the analysis. The cell membrane region was manually segmented for each cell, and FRET efficiency was calculated only from the cell membrane.

Supplementary References

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Supplementary Figure Legends

Figure S1: Validation of immunoblotting quantification method. A: Control immunoblot from whole-cell lysate of γ 2A cells transiently transfected with JAK2-HA WT or JAK2-HA V617F as indicated. Lysates were run at different dilutions in lysis buffer to gauge linearity of immunoblotting signal. For detection, immunoblot is cut into three pieces (indicated with dashed lines) and the pieces double stained with Anti-pJAK2 + Anti-HA or Anti-pSTAT1 + STAT1, or single-stained with Anti-Actin. B: Example of quantification procedure using ImageStudio software (LICOR Biosciences). Shown is an example area from the immunoblot shown in (A) and indicated by a white dotted line. Bold blue boxes are the manually assigned areas of interest, each encompassing a single band (note, e.g., that for STAT1, only STAT1 α is quantified). The light blue boxes on top and below each band are used to calculate the local background from the lane. From the background, the median signal intensity is used as a background value, which is deducted from the total signal from the area of interest. C: pJAK2 and total JAK2-HA quantifications showing the approximate linearity of the signal over the measured intensity range. Last panel shows the ratio of pJAK2 and JAK2-HA signal intensities, which is used as a measure of phosphorylation status ("Normalized pJAK2"). The point of deviation from linearity on the normalized pJAK2 panel shows the limits of pJAK2+JAK2-HA quantifiability (down to pJAK2 signal intensities of ~50), thus limiting the direct comparison of pJAK2 values to samples with relatively strong pJAK2 signals. D: pSTAT1 and total STAT1 quantifications as delineated above for (C). Note the exceptional linearity of STAT1 signals (and the ensuing stability of the normalized pSTAT1 value in the last panel), thus rendering pSTAT1 better suited for comparison of samples over a wide range of pSTAT1 signal intensities. E: Quantification of Actin signals showing poor linearity over the tested range. Thus Actin was not used for normalization in quantification of immunoblot data.

Figure S2: Characterization of effects of suppressing mutation on cytokine signaling in JAK2 WT background. Related to Figure 4. A and B: Immunoblots related to Figure 4 A and B, respectively. Immunoblots of whole-cell lysates from γ 2A cells transiently transfected with full-length JAK2-HA (and mutants thereof), STAT5-HA, and EPOR-HA (A) or full-length JAK2-HA mutants only (B), and stimulated with the indicated amount of cytokine for 30 min. Quantification of immunoblots was done as shown in Figure S1. C: Related to Figure 4 E, qPCR of *IRF1* from RNA extracted from γ 2A cells transiently transfected with JAK2-HA mutants and stimulated with EPO for 2 h showing the specificity of *IRF1* induction. D: Immunoblots of whole-cell lysates from γ 2A cells transiently transfected with full-length JAK2-HA mutants and stimulated with 1 μ g/ml IFN γ for the indicated times before lysis. JAK2-HA E592R and F537A show no induction of pSTAT1 even at longer stimulation times.

Figure S3: JAK2 activation by V617F relies on the same interfaces to activate as JAK2 activation by cytokine. Suppression calculated as STAT5 or STAT1 reporter activity relative to basal activity of JAK2 V617F (y-axes) and stimulated wild-type JAK2 (10 U/ml EPO or 5 ng/ml IFN γ ; x-axes). Data for figure is from the same experiments as for Figures 2 and 3.